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Transport of citrate catalyzed by the sodium-dependent citrate carrier of *Klebsiella pneumoniae* is obligatorily coupled to the transport of two sodium ions

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Aerobically grown *Escherichia coli* GM48 harboring plasmid pKScitS that codes for the sodium-dependent citrate carrier from *Klebsiella pneumoniae* (CitS) allows initial-rate measurements of citrate uptake in whole cells. The cation stoichiometry and selectivity of CitS was studied using this experimental system. The relationship between the initial rate of uptake of citrate and the Na^+ concentration was sigmoidal at pH values between 5 and 7 suggesting a Na^+ stoichiometry higher than 1. Rates of uptake increased quadratically in a range of non-saturating Na^+ concentrations showing that two Na^+ are translocated/catalytic cycle. Symport of Na^+ is absolutely required in the range pH 5–7 because no uptake could be detected in the absence of Na^+ . Protons cannot replace Na^+ in the translocation step but the decrease in apparent affinity for Na^+ towards lower pH suggests that protons can compete with Na^+ for the cation-binding sites.

Li^+ can replace Na^+ in the symport reaction but it takes about a 200-fold higher concentration of Li^+ over Na^+ to achieve the same rate of uptake, showing that the affinity of CitS for Li^+ is much lower than for Na^+ . Though high Li^+ concentrations have an inhibitory effect on citrate uptake, the data suggest that the Li^+ stoichiometry is also 2.

Chemiosmotic energy transduction in bacteria can depend on both proton and sodium ion cycling across the cytoplasmic membrane. Cells maintain an inward directed proton motive force (pmf) and sodium motive force (smf) across the cell membrane. The pmf is generated by primary proton pumps, the smf either, directly, by primary Na^+ pumps or, indirectly, through Na^+/H^+ antiport activity. Secondary solute transporters use the free energy stored in the ion gradients to accumulate a solute inside the cell. Mechanistically, this implies that the solute is transported in symport with H^+ or Na^+ . A large group of secondary transporters has been described that exclusively couple the translocation of solute with H^+ , e.g. most of the sugar permeases in *Escherichia coli* [1, 2]. In extremophiles many solute transporters are exclusively driven by the Na^+ gradient, e.g. all amino acids in the halophile *Micrococcus halobius* [3]. The difference in coupling ion must reflect differences in the cation-binding site on the carrier protein. In addition to the two classes of permeases that differ in the cation usage, a number of transporters have been described that use either H^+ or Na^+ as the coupling ion. For instance, the melibiose carrier of *E. coli* encoded by the *melB* gene transports melibiose in symport with a single cation that can be either H^+ , Na^+ or Li^+ [4, 5]. The different cations compete for the cation-binding site on the permease indicating a lower selectivity of the binding

site. In cases where more than one coupling ion is transported/catalytic cycle, a dependency on both the H^+ and Na^+ gradient could reflect separate highly selective binding sites for H^+ and Na^+ or competition for the cation-binding sites between H^+ and Na^+ as observed with MelB. The Na^+ -dependent citrate transporter of *Klebsiella pneumoniae* is an example of such a transport protein.

K. pneumoniae grown aerobically expresses a citrate transporter that is proton dependent (CitH), whereas under anaerobic growth conditions a different citrate transporter is expressed that is sodium-ion dependent (CitS) [6, 7]. Both the genes coding for these permeases have been cloned and sequenced [8, 9]. The enzymes have been characterized after expression in *E. coli* which is the perfect host for citrate permease genes since it does not contain chromosomally encoded citrate-transport systems. It was demonstrated that the divalent anionic state of citrate Hcit^{2-} is the transported particle [10, 11]. Citrate uptake in membrane vesicles containing either CitS or CitH could be driven solely by a transmembrane electrical potential gradient (negative inside), indicating that at least three cations are transported in symport with Hcit^{2-} . Uptake of citrate in membrane vesicles containing CitH could be driven by an artificial pH gradient indicating that the symported cations were protons [10]. In case of membrane vesicles containing CitS, both a chemical H^+ gradient and chemical Na^+ gradient could drive citrate accumulation [9]. Therefore, the coupling ions for CitS are H^+ and Na^+ . Here, we report on the Na^+ stoichiometry of CitS of *K. pneumoniae*. It will be demonstrated that two Na^+ are transported/catalytic cycle and that the Na^+ -binding sites are highly selective for Na^+ .

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Abbreviations. pmf, proton motive force; smf, sodium ion motive force; CitH, proton-dependent citrate carrier; CitS, sodium-ion-dependent citrate carrier.

EXPERIMENTAL PROCEDURES

Materials

[1,5-¹⁴C]Citrate (111 mCi/mmol) was obtained from Amersham Radiochemical Center. KH₂PO₄ (low Na⁺) and KOH (low Na⁺) were from Merck. All other chemicals were reagent grade and obtained from commercial sources.

Plasmids

Constructs pGR3 and pRS63-2 containing the coding sequences for CitH and CitS have been described before [8, 9]. A fragment containing the *citS* gene was restricted from pRS63-2 by a digestion with *Bam*HI and *Hind*III and subsequently ligated into the multiple cloning site of the vector pBlueScript II KS(-), resulting in plasmid pKScitS. Similarly, plasmid pKScitH was constructed by ligating the *Hind*III–*Nhe*I fragment of pGR3 into the *Hind*III–*Xba*I sites of the same vector. In both vectors expression of the genes is under control of the lac promoter on the pBluescript vector.

Cell growth

Plasmids pKScitS and pKScitH were transformed to *E. coli* strain GM48. Transformants were examined for citrate metabolism on Simmons citrate agar (Difco) supplemented with 50 µg/ml carbenicillin. Stocks of the transformed cells were stored in glycerol at –70°C until use. Cells from the stocks were grown overnight on Luria-Bertani agar plates (50 µg/ml carbenicillin) after which the plate was stored at 4°C. Cells from the plate were transferred to 50 ml Luria-Bertani medium supplemented with 50 µg/ml carbenicillin and grown under conditions of continuous shaking until an absorbance at 650 nm of about 0.8. GM48/pKScitS was grown in the absence of isopropyl-thio-β-D-galactopyranoside and at 37°C whereas GM48/pKScitH was grown in the presence of 0.5 mM isopropyl-thio-β-D-galactopyranoside and at 30°C. These conditions resulted in optimal levels of expression for the kinetics studies. Nevertheless, the levels of expression differed significantly between the different batches of cells. The level of expression had no effect on the kinetics analysis described in this study. Cells were harvested and washed three times with 50 ml low-Na⁺ KP_i of the appropriate pH. Cells were resuspended to an absorbance at 650 nm of 10 and stored on ice until use. Citrate uptake in these cells did not decrease significantly for at least three days.

K. pneumoniae was grown in minimal-citrate medium [12]. A 500-ml serum bottle filled to the top with medium was inoculated with cells and closed tightly. Cells were allowed to grow overnight at 37°C without shaking. Cells were harvested, washed and stored as above.

Cell protein was determined according to Lowry et al. [13] using bovine serum albumin as a standard.

Uptake assays

Cells were resuspended in the appropriate buffer in a total volume of 95 µl and incubated for 10 min at 30°C. 5 µl [1,5-¹⁴C]citrate was added to a final concentration of 5 µM at *t* = 0. Initial-rate measurements implied the measurement of citrate uptake at four consecutive time points with intervals of 10 s. Uptake was stopped by adding 2 ml ice-cold 100 mM LiCl followed by rapid filtration over 0.45-µm nitrocellulose filters. Filters were washed once with 2 ml of the LiCl solu-

tion and submerged immediately in scintillation fluid to prevent any further metabolism of citrate inside the cells on the filter. After the filters were dissolved, the trapped radioactivity was measured in a liquid-scintillation counter. The amount of radioactivity bound unspecifically to the filter was determined by adding the quenching solution to a sample of cells before the radioactive citrate, followed by filtering and washing. The background amounted to about 0.3% of the total label present in a sample (≈110×10³ cpm).

Citrate metabolism assay

Cells were resuspended in 190 µl 40 mM KP_i/10 mM NaP_i pH 6 and incubated for 10 min at 30°C. A 10-µl aliquot of [1,5-¹⁴C]citrate was added to give a final concentration of 5 µM. The assay tube was vortexed intermittently to facilitate the release of radioactive CO₂. Samples of 10 µl were withdrawn at the indicated time points and added immediately to 2 ml scintillation fluid. The CO₂ production was followed by the decrease of radioactivity in the sample. The radioactivity present in the sample at the zero time point was evaluated from the radioactivity present in 10 µl 5 µM [1,5-¹⁴C]citrate treated identically.

Analysis of the data

The stoichiometric coefficient (*n*) for Na⁺ associated with citrate transport catalyzed by CitS was determined by fitting the data set comprising internalized label (cpm) at different time points (*t*) and at different Na⁺ concentrations ([Na⁺]) to the following equation by a non-linear fitting procedure:

$$\text{cpm} = \text{cpm}_0 + (v_0 + k[\text{Na}^+]^n)t \quad (1)$$

cpm₀ is the background of the assay procedure. *v*₀ is a very low apparent rate that is due to a time-dependent adsorption phenomenon that is not related to the presence of a citrate carrier in the membrane. Finally, *k* is a pseudo-second-order-rate constant. Eqn (1) allows the determination of the kinetic parameters without any manipulation of the raw data. Apparent rate *v*₀ was also measured experimentally with cells that did not harbor the plasmids coding for the citrate carriers and did not differ significantly from the fitted values.

Simulation of kinetic behavior

The kinetics of an enzyme catalyzing symport of a substrate and two Na⁺ was simulated using the CACES program [14]. The simulations were set up to analyze the Na⁺ dependency of the turnover rate. The kinetic scheme consisted of 16 states and described a carrier protein with one binding site for citrate and two binding sites for Na⁺. The binding sites were oriented either to the cytoplasmic side or the periplasmic side of the membrane. The orientation of the three binding sites was the same at any point in time. The order of binding was taken to be random. The association and dissociation rate constants for Na⁺ binding were 10 mM^{–1} s^{–1} and 40 s^{–1} (*K*_d = 4 mM), respectively. The association and dissociation rate constants for citrate binding were 10 µM^{–1} s^{–1} and 10 s^{–1} (*K*_d = 1 µM), respectively. Translocation of the binding sites was allowed with all three sites empty or occupied. The back and forward rate constants of the translocation equilibria were 5 s^{–1}. Turnover rates were simulated with internal citrate and Na⁺ concentrations set to zero.

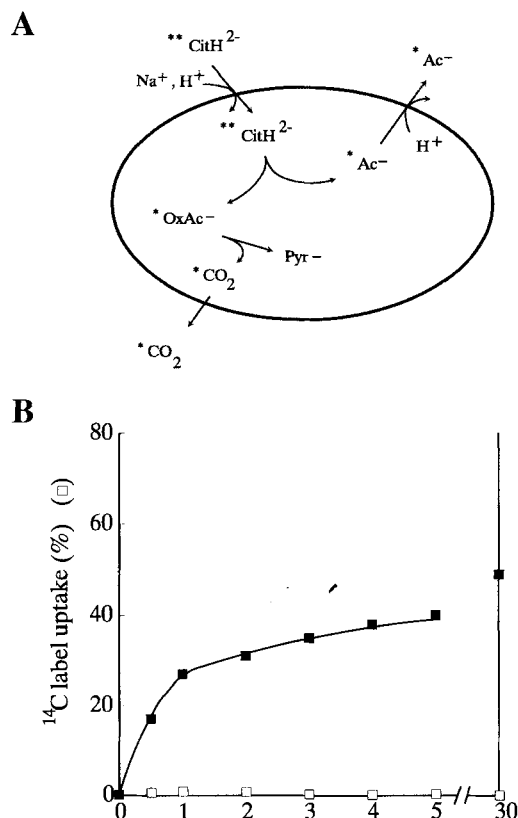


Fig. 1. Uptake and metabolism of citrate by whole cells of *K. pneumoniae*. (A) Metabolic pathway for citrate (Cit) in anaerobically grown *K. pneumoniae* showing the fate of the two labeled C atoms (indicated by the asterisks) originally present in the citrate molecule. OxAc, oxaloacetate; Pyr, pyruvate. (B) Percentage of label inside the cells (□) and released as radioactively labeled $^{14}\text{CO}_2$ (■). The protein concentration was 0.3 mg/ml. The metabolic pathway in (A) shows that 50% of the label originally in citrate should be recovered in radioactively labeled CO_2 which is in fair agreement with the experimental results.

RESULTS

Citrate uptake and metabolism by whole cells

Anaerobically grown cells of *K. pneumoniae* catalyze citrate uptake by citS in symport with H^+ and Na^+ (Fig. 1). Internalized citrate is cleaved by citrate lyase to yield oxaloacetate and acetate. The latter may leave the cell as acetic acid, thereby recycling a proton previously used in the uptake of citrate. Oxaloacetate is cleaved by a membrane bound decarboxylase that conserves the free energy of the decarboxylation reaction by pumping Na^+ across the membrane [15]. The smf formed serves to drive citrate uptake. Citrate metabolism is much faster than citrate uptake (Fig. 1). No significant radioactivity could be detected inside the cells upon addition of [1,5- ^{14}C]citrate to the cells. Nevertheless, the production of [^{14}C] CO_2 reveals the activity of the transporter which, apparently, is rate limiting in the metabolic pathway [16]. These observations reveal that whole cells of *K. pneumoniae* cannot be used to study the kinetics of the sodium-dependent citrate transporter.

Plasmid pKScitS harbors the gene coding for the sodium-dependent citrate carrier of *K. pneumoniae*. It allows the expression of CitS in *E. coli* grown under aerobic conditions. [1,5- ^{14}C]Citrate entering the cells is completely converted to [^{14}C] CO_2 in the citric acid cycle (Fig. 2). The distribution of

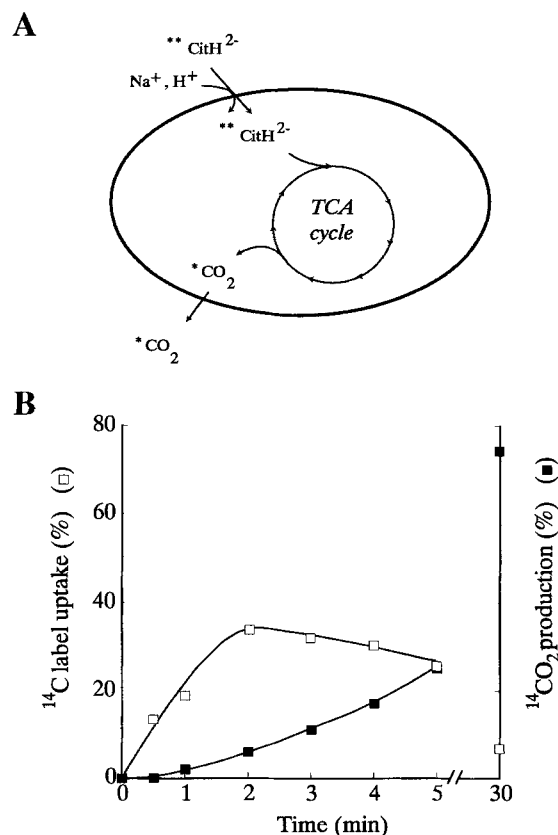


Fig. 2. Uptake and metabolism of citrate by whole cells of *E. coli* GM48/pKScitS. (A) Metabolic pathway for citrate in aerobically grown *E. coli* showing the fate of the two labelled C atoms originally present in the citrate molecule. (B) Uptake of radioactive label (□) and the production of radioactively labeled CO_2 (■). The protein concentration was 0.3 mg/ml. All radioactivity originally in the citrate (Cit) should eventually be recovered as [^{14}C] CO_2 .

the internalized label over the citric-acid-cycle intermediates prevents the production of significant amounts of [^{14}C] CO_2 during the first minute after addition of labeled citrate to the cells (Fig. 2). The formation of the internal pools is shown by a significant uptake of label by the cells that increases linearly during the first minute (see also Fig. 5). Therefore, aerobically grown *E. coli* cells expressing CitS provide a suitable system to measure initial rates of citrate uptake catalyzed by CitS.

Na^+ dependence of the rate of citrate uptake

The initial rate of citrate uptake in *E. coli* GM48 cells harboring plasmid pKScitS was measured in 50 mM phosphate, pH 6, composed of mixtures of NaP_i and KP_i (Fig. 3). Resting cells of *E. coli* maintain a pmf across the cell membrane at the expense of endogenous energy reserves. The effect of increasing amounts of NaP_i in the buffer on the ion gradients across the cytoplasmic membrane that drive citrate uptake was investigated by repeating the same experiment with cells harboring plasmid pKScitH that contains the gene coding for the proton-dependent citrate carrier of *K. pneumoniae* (CitH). CitH is driven by the pmf, but the ΔpH component is much more effective in driving citrate uptake than the $\Delta\psi$ component [10]. Therefore, citrate uptake by CitH is not only sensitive to the magnitude of the pmf but also to the distribution over the pH gradient and the transmembrane

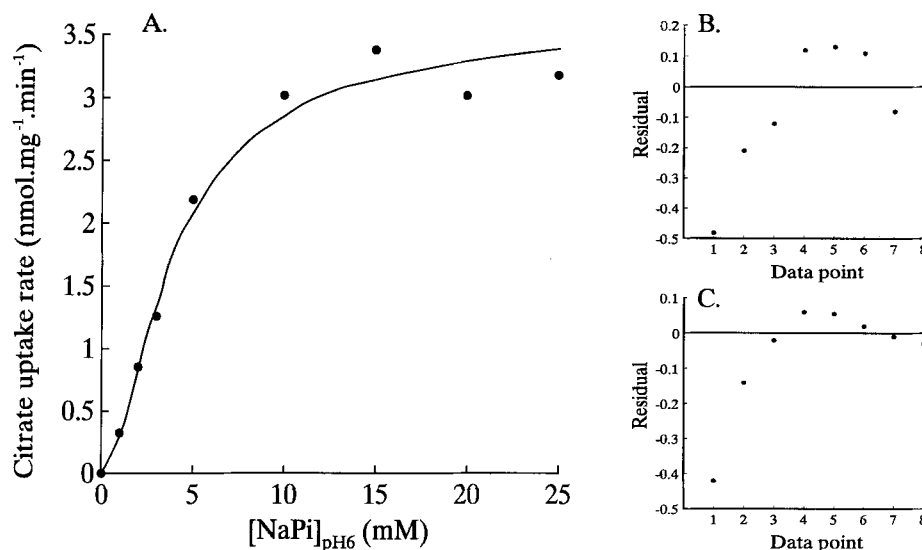


Fig. 3. The Na⁺-concentration dependency of the initial rate of citrate uptake in *E. coli* GM48/pKScitS. (A) The rate of citrate uptake was measured in 50 mM phosphate pH 6 consisting of mixtures of KP_i and NaP_i (●). The protein concentration was 0.3 mg/ml. The solid line is the result of a simulation of a kinetics scheme representing a carrier that catalyzes symport of citrate with two Na⁺. The ordinate was arbitrarily expanded to match the experimental data points. (B, C) Residual analysis after fitting the experimental (B) and simulated (C) data to a Michaelis-Menten curve assuming a Na⁺ stoichiometry of 1. The data points represent Na⁺ concentrations of 1, 2, 3, 5, 10, 15, 20 and 25 mM. The mean residual errors are 15% and 8.5% for the experimental and simulated data, respectively.

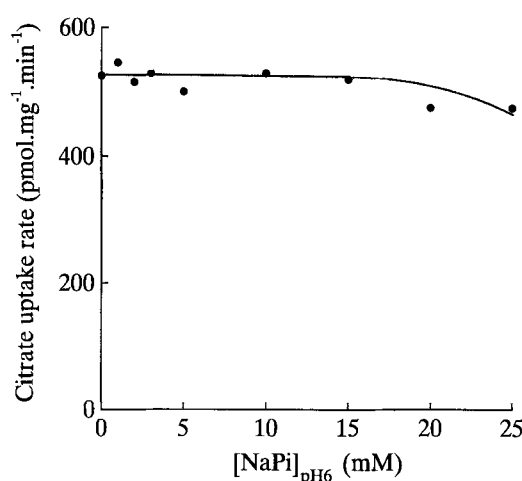


Fig. 4. The Na⁺ concentration dependency of the initial rate of citrate uptake in *E. coli* GM48/pKScitH. See the legend to Fig. 3 for experimental details. The protein concentration was 1.5 mg/ml.

electrical gradient. Fig. 4 shows that the initial rate of citrate uptake catalyzed by CitH is independent of the Na⁺ concentration up to about 10 mM and starts decreasing slowly at higher concentrations. In the range 0–25 mM Na⁺ neither the magnitude nor the composition of the pmf changes dramatically. The magnitude of the membrane potential under the conditions of the experiment described in Figs 3 and 4 was 68 ± 5 mV and the internal pH was 7.2 ± 0.2 . In addition to the pmf, uptake of citrate catalyzed by the Na⁺-dependent citrate carrier (CitS) is driven by the Na⁺ gradient [9]. In the presence of Na⁺, a Na⁺ gradient will equilibrate with Δ pH and $\Delta\psi$ through the activities of the sodium/proton antiporters in the membrane of *E. coli* [17]. In conclusion, the driving force on CitS is more or less constant when the Na⁺ concentration in the buffer is increased from 0 to 25 mM

and, therefore, the changes in the rates presented in Fig. 3 reflect the kinetics of the carrier in relation to the Na⁺ concentration.

Fig. 3A shows that no citrate uptake could be detected in the absence of Na⁺ and that the uptake rate saturates above about 10 mM NaP_i. The apparent affinity constant is about 3.5 mM. The data was fitted to a Michaelis-Menten type of saturation curve using a non-linear fitting procedure resulting in a mean residual error of 15%. Analysis of the residuals reveals a structured deviation of the experimental data points from the calculated data points indicating that the experimental data is not well described by a Michaelis-Menten curve (Fig. 3B). Analysis of the data with a Michaelis-Menten type of relation assumes that one Na⁺ is translocated in symport with citrate. The sigmoidal solid line in Fig. 3A represents a simulation of the experiment based upon a kinetics scheme for a citrate carrier that catalyzes citrate uptake in symport with two Na⁺ (see Experimental Procedures). Fitting of the simulated data to the Michaelis-Menten equation results in a non-random distribution of the residuals with a structure very similar to that observed with the experimental data (Fig. 3C). The analysis suggests that citrate uptake by CitS is in symport with two Na⁺.

Na⁺-dependent kinetics under non-saturating conditions

An enzyme-catalyzed reaction behaves kinetically similar to any chemical reaction under conditions when no saturation of the enzyme occurs. The rate equation with respect to one of the substrates, i.e. Na⁺, then takes the form

$$v \approx [\text{Na}^+]^n \quad (2)$$

in which n is the order of the reaction in Na⁺, or the number of Na⁺ translocated/catalytic cycle. Fig. 5 shows the uptake of citrate catalyzed by CitS at Na⁺ concentrations up to 0.5 mM. At this concentration the rate is approximately 4% of the maximal rate. In the absence of Na⁺ the observed rate is insignificant, indicating that Na⁺ is required for transport

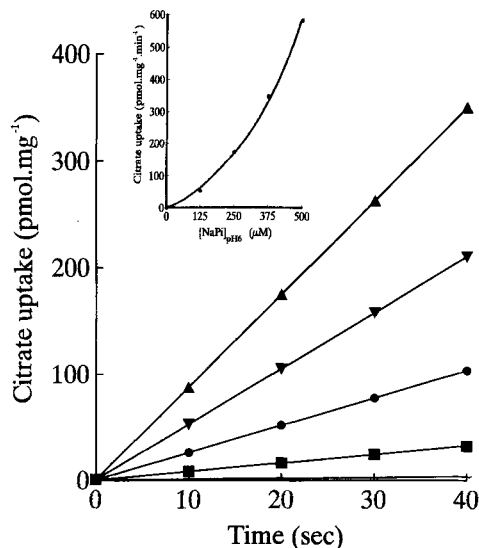


Fig. 5. Kinetics of citrate uptake in *E. coli* GM48/pKScitS at low Na^+ concentrations. Citrate uptake was measured in 50 mM phosphate, pH 6, in the presence of 0 (+), 125 (■), 250 (●), 375 (▼) and 500 μM (\blacktriangle) NaPi , pH 6. The protein concentration was 1.5 mg/ml. The insert shows the rate of citrate uptake as a function of the NaPi concentration.

catalyzed by CitS. The data set shown in Fig. 5 was analyzed as described under Experimental Procedures, resulting in a value of 1.79 for n in Eqn (1) with a mean residual error of 3.1%. The almost quadratic relation between the initial rate of uptake and the Na^+ concentration is evident from the insert in Fig. 5. A theoretical value of $n = 2$ for the symport of two Na^+ will only be found when the rates are measured at infinitely low Na^+ concentrations. To establish the effect of some saturation on the value of n , the experiment was simulated using the same scheme that resulted in a reasonable fit to the experimental data in Fig. 3. The calculated rates in the Na^+ concentration range from 0 to 0.5 mM were fitted to Eqn (2), resulting in a value of $n = 1.84$ with a mean residual error of 1.4% which is in good agreement with the experimental value. It is concluded that under these conditions two Na^+ are translocated/catalytic cycle of CitS.

In the experiment shown in Fig. 5, the cells were previously incubated with the indicated concentrations of sodium phosphate. Therefore, an inwardly directed Na^+ gradient will have developed in response to the appropriate components of the pmf. The effect of internal Na^+ concentration on the kinetics was investigated by adding labeled citrate together with sodium phosphate to cells previously incubated with potassium phosphate. The observed rates were not significantly different as those reported in Fig. 5, indicating that the low internal Na^+ concentrations after equilibration do not effect the rate of uptake (data not shown). Fitting of the data set to Eqn (1) resulted in a value for n of 1.7 with a mean residual of 10%.

Competition between Na^+ and H^+

The ability of CitS to translocate citrate both with H^+ and Na^+ was investigated by measuring the initial rates of citrate uptake at both a lower and a higher pH (Fig. 6). At pH 5 the relationship between the rate and the Na^+ concentration is clearly sigmoidal. The apparent affinity for Na^+ has decreased relative to the affinity at pH 6. The Na^+ concentrations giving half-maximal rates are approximately 8 mM and 3.5 mM, respectively. Taking into consideration that the Na^+ concentration at pH 7 is 1.5-times as high as the P_i concentration, the shape of the curve at pH 7 is not very different from the one at pH 6. The kinetics characteristics at the three pH values allow the determination of the Na^+ stoichiometry at low Na^+ concentrations (Fig. 7). At none of the pH values could a significant rate of citrate uptake be detected in the absence of Na^+ . Furthermore, the quadratic increase of the rate with increasing Na^+ concentration observed before at pH 6 was retained at pH 5 and pH 7. The results reveal that transport of citrate catalyzed by CitS is obligatorily coupled to the transport of two Na^+ in the pH range 5–7.

Competition between Li^+ and Na^+

Li^+ and Na^+ have been reported to be equally good coupling ions for the sodium-dependent citrate permease of *K. pneumoniae* when expressed in *E. coli* [7]. Table 1 shows the expected quadratic increase in the rate of citrate uptake by *E. coli*/pKScitS in the presence of 150 μM and 300 μM

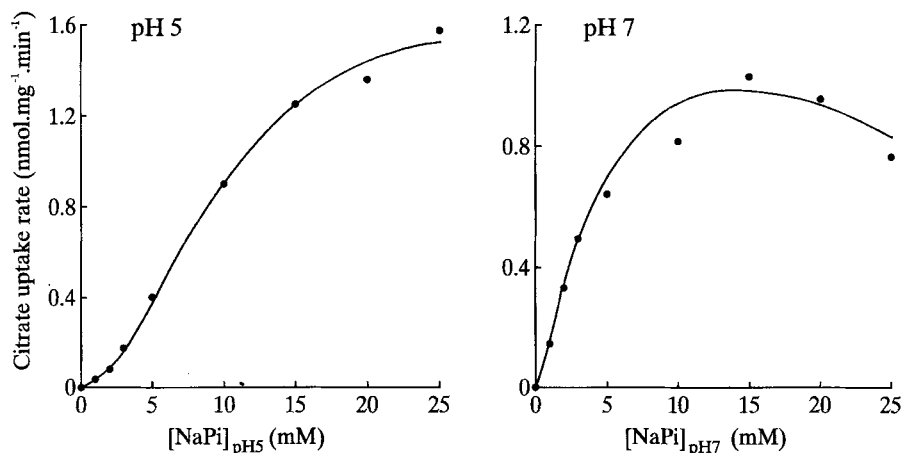


Fig. 6. The Na^+ -concentration dependency of the initial rate of citrate uptake at pH 5 and pH 7 in *E. coli* GM48/pKScitS. Citrate uptake was measured in 50 mM phosphate of the indicated pH consisting of mixtures of KP_i and NaPi . The protein concentration was 0.6 mg/ml in both experiments.

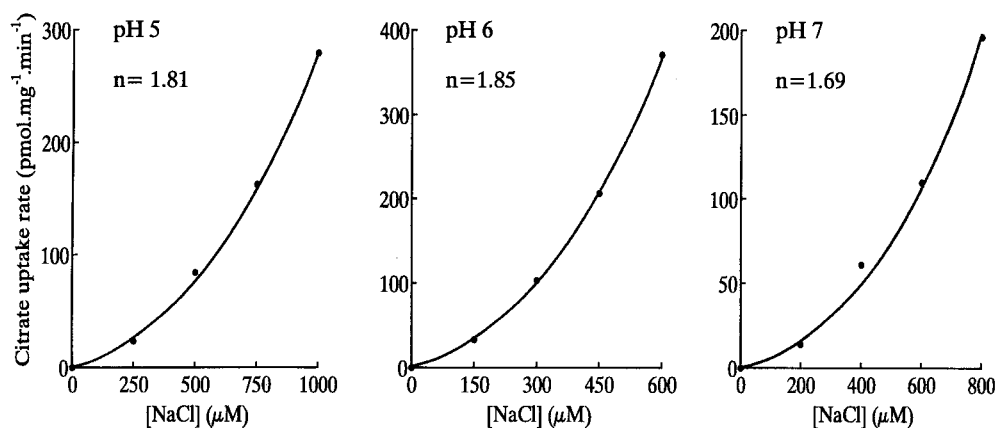


Fig. 7. The Na^+ stoichiometry at pH values of 5, 6 and 7. Citrate uptake was measured in 50 mM phosphate of the indicated pH supplemented with mixtures of KCl and NaCl resulting in a constant Cl^- concentration. The protein concentrations were 2.2, 1.65 and 1.75 mg/ml at pH 5, 6 and 7, respectively. The five time curves obtained at each pH were fitted to Eqn (1) and the resulting values for n are indicated in the plots that represent the rate dependency of the Na^+ concentration at the three pH values. The mean residual error from the fit were 7%, 10% and 7% for pH 5, 6 and 7, respectively.

Table 1. Competition between Na^+ and Li^+ ions. Citrate uptake was measured in 40 mM potassium phosphate, pH 6, plus the indicated concentrations of cations that were added as the chloride salt. Cell protein in the experiments with the Na^+ -dependent (CitS) and the proton-dependent (CitH) citrate carrier were 1.8 mg/ml and 0.75 mg/ml, respectively.

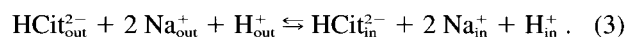
Concentration of			Citrate uptake rate with	
Na^+	Li^+	K^+	CitS	CitH
mM			$\text{pmol} \cdot \text{mg}^{-1} \cdot \text{min}^{-1}$	
0	0	60	<3	363
0.15	0	60	31	348
0.3	0	60	101	371
0	30	30	39	304
0	60	0	85	215
0.15	30	30	73	315

NaCl. To achieve the same rate of uptake in the presence of Li^+ a 200-fold higher concentration of LiCl was needed. Raising the LiCl concentration from 30 mM to 60 mM resulted in a twofold increase of the rate. However, the control experiments with *E. coli*/pKScitH expressing the proton-dependent citrate carrier showed a similar progressive reduction of the rate of citrate uptake with LiCl as observed for high concentrations of Na^+ (see Fig. 4). Therefore, the twofold increase of the rate catalyzed by CitS upon doubling of the Li^+ concentration is an underestimation and the Li^+ stoichiometry is probably two as well. The combination of 150 μM NaCl and 30 mM LiCl results in a rate that is equal to the sum of the rates when the two ions are present separately. This reflects a low affinity of CitS for Li^+ relative to Na^+ rather than an inhibition of the translocation by Li^+ .

DISCUSSION

In general, the activities of enzymes follow a pH profile characterized by a pH optimum. For enzymes like proton symporters which have protons as substrate, this property makes it difficult to establish experimentally the proton-con-

centration dependency of the enzyme. In case the substrate of the carrier is a weak acid, e.g. citrate, this is even more complicated because any change of the proton concentration results in a change of the protonation state of the acid. Symporters that use Na^+ as the coupling ion do not suffer from these drawbacks and allow the characterization of the symport reaction with respect to the cation. In this study we have taken advantage of this situation by measuring the initial rate of citrate uptake at different Na^+ concentrations catalyzed by the Na^+ -dependent citrate carrier of *K. pneumoniae* expressed in *E. coli*. The relation between the initial rate of citrate uptake and the Na^+ concentration proved to be sigmoidal in the pH range 5–7 indicating a stoichiometric coefficient for Na^+ higher than 1. Measurement of the relationship between the uptake rate and Na^+ concentration under non-saturating conditions indicated transport of two Na^+ /catalytic cycle. Though bacterial secondary transporters are believed to be active as monomers [18, 19], the data could be explained by a dimer exhibiting cooperativity between the monomeric subunits such that each would catalyze citrate transport in symport with one Na^+ . This would result in a citrate: Na^+ stoichiometry of 1:1. However, the cooperativity would probably also be reflected in the citrate-dependent kinetics, which was not observed in the pH range 5.5–7 [11]. Provided that the citrate carrier functions as a monomer, we conclude that the citrate: Na^+ stoichiometry is 1:2 and that the reaction catalyzed by CitS is



Dimroth and Thomer [7] argued that it would be likely that two Na^+ would be translocated in symport with citrate by CitS since this would complete the sodium cycle in *K. pneumoniae*. The two Na^+ pumped out of the cell by the oxaloacetate decarboxylase downstream in the metabolic pathway of citrate under anaerobic conditions would be recycled by the uptake of citrate. However, in a subsequent study [16] they interpreted their experimental results with a stoichiometry of three Na^+ in contradiction to the results presented here. Their conclusion was based upon their inability to show proton movement concomitantly with citrate transport using the fluorescent probe 9-amino-6-chloro-2-methoxyacridine. However, van der Rest et al. [11] clearly demonstrated that a proton gradient can drive citrate uptake

by CitS in right-side-out membrane vesicles. Possibly the fluorescence technique was not sensitive enough to detect the generated pH differences. An attempt was made to measure the $\text{Na}^+:\text{citrate}$ stoichiometry by measuring, in parallel, the rate of efflux of $^{22}\text{Na}^+$ and $[^{14}\text{C}]\text{citrate}$ from loaded membrane vesicles prepared from *E. coli* cells in which CitS was expressed. The results indicated a $\text{Na}^+:\text{citrate}$ stoichiometry of 1, also at variance with the present results [11]. The difference is probably explained by the high background rates observed in the absence of the second substrate, i.e. Na^+ efflux in the absence of citrate and citrate efflux in the absence of Na^+ , which makes the measurements less reliable.

The coupled translocation of HCit^{2-} , H^+ and the two Na^+ (Eqn 3) requires the formation of a complex between the carrier and the four substrates before the binding sites on the enzyme can reorient, thereby exposing the substrates to the other side of the membrane. Competition between different cations for the cation-binding sites may lead to decreased transport activity when binding of the competing ion(s) does not result in a translocation-competent state of the carrier. No uptake of citrate will occur when only the competing ion is present. In the presence of the competing ion, the apparent affinity will be lower for the functional ion. Alternatively, the complex of the carrier and the competing ion can also isomerize and the rate of citrate uptake may increase or decrease depending on the affinities and translocation-rate constants for the two ions. Citrate uptake will occur with either cation in the absence of the other and broken stoichiometries may be found when measured in the presence of both cations. The present results show that, no citrate uptake could be detected in the absence of Na^+ ; the stoichiometry coefficient for Na^+ did not change over a 100-fold proton-concentration range (from pH 5 to pH 7); the apparent affinity for Na^+ decreased with increasing proton concentration; citrate uptake could be measured in the presence of Li^+ alone. These results show that Na^+ cannot be replaced by H^+ in the translocation step, but, in addition, they suggest that H^+ may bind competitively to the Na^+ -binding sites on the carrier. Li^+ can replace Na^+ in the translocation step, but the affinity of the Na^+ -binding sites for Li^+ is very low. The high concentrations of Li^+ necessary for uptake of citrate do not allow a more detailed characterization of the $\text{Li}^+/\text{citrate}$ symport reaction due to secondary effects on the state of energization

of the cells in the presence of high Na^+ or Li^+ concentrations.

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REFERENCES

1. Poolman, B. & Konings, W. N. (1993) *Biochim. Biophys. Acta* **1183**, 5–39.
2. Henderson, P. J. F. (1991) *Curr. Opin. Struct. Biol.* **1**, 590–601.
3. Nikolaev, Y. A., Matveeva, N. I. & Plakunov, V. K. (1990) *Microbiology (Washington DC)* **59**, 213–221.
4. LeBlanc, G., Pourcher, T., Bassilana, M. & Deckert, M. (1991) in *Ion coupled sugar transport in microorganisms* (Page, M. & Henderson, P. J. F., eds) pp. 183–191, CRC Uniscience, Sery.
5. Wilson, D. M. & Wilson, T. H. (1987) *Biochim. Biophys. Acta* **904**, 191–200.
6. Schwarz, E. & Oesterhelt, D. (1985) *EMBO J.* **4**, 1599–1603.
7. Dimroth, P. & Thomer, A. (1986) *Biol. Chem. Hoppe-Seyler* **376**, 813–823.
8. van der Rest, M. E., Schwarz, E., Oesterhelt, D. & Konings, W. N. (1990) *Eur. J. Biochem* **189**, 401–407.
9. van der Rest, M. E., Siewe, R. M., Abee, T., Schwarz, E., Oesterhelt, D. & Konings, W. N. (1992) *J. Biol. Chem.* **267**, 8971–8976.
10. van der Rest, M. E., Abee, T., Molenaar, D. & Konings, W. N. (1991) *Eur. J. Biochem* **195**, 71–77.
11. van der Rest, M. E., Molenaar, D. & Konings, W. N. (1992) *J. Bacteriol.* **174**, 4893–4898.
12. Dagley, S. (1969) *Methods Enzymol.* **13**, 160–163.
13. Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265–275.
14. Lolkema, J. S. (1993) *J. Biol. Chem.* **268**, 17850–17861.
15. Dimroth, P. (1987) *Microbiol. Rev.* **51**, 320–340.
16. Dimroth, P. & Thomer, A. (1990) *J. Biol. Chem.* **265**, 7721–7724.
17. Schuldiner, S. & Padan, E. (1992) in *Alkali cation transport systems in prokaryotes* (Bakker, E. P., ed.) pp. 25–51, CRC Press, Boca Raton.
18. Kaback, H. R. (1990) in *The bacteria* (Krulwich, T. A., ed.) vol. XII, pp. 151–202, Academic Press Inc., New York.
19. Ambudkar, S. V., Anantharam, V. & Maloney, P. C. (1990) *J. Biol. Chem.* **265**, 12287–12291.